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Novel iminobenzoxathiolone compound inhibits nuclear factor-κB activation targeting inhibitory κB kinase β and down-regulating interleukin-1β expression in lipopolysaccharide-activated macrophages

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ABSTRACT

Benzoxathiolone derivatives have been reported to show pharmacological potentials in the psoriasis and acne. However, molecular basis for these pharmacological properties is little known. We postulated that the derivatives could mediate some of their pharmacological actions by modulating nuclear factor (NF)-κB activation, which is closely linked to the inflammatory and immune disorders. In this study, a novel iminobenzoxathiolone LYR-71 of 6-methyl-2-propylimino-6,7-dihydro-5*H*-benzo[1,3]oxathiol-4-one has been demonstrated to inhibit *in vitro* catalytic activity of inhibitory κB (IκB) kinase β (IKKβ), a key enzyme required for NF-κB activation, with an IC_{50} value of 7 μM. LYR-71 inhibited IKKβ-mediated phosphorylation of cytoplasmic IκBα in lipopolysaccharide (LPS)-activated macrophages, and sequentially preventing IκBα degradation as well as transcriptional activation of NF-κB. Furthermore, LYR-71 down-regulated LPS-induced transcription of interleukin (IL)-1β or other cytokines in the cells, and inhibited expression vector IKKβ-elicited IL-1β promoter activity. Taken together, LYR-71 was an efficient inhibitor of IKKβ, preventing NF-κB activation in macrophages, and this mechanism of action could contribute its down-regulatory effect on LPS-induced expression of inflammatory cytokines at the transcription level.

1. Introduction

Nuclear factor (NF)-κB regulates a central common signaling for immunity, cell proliferation and survival, and thus its modulating agents are widely considered to possess pharmacological potentials in the inflammatory and proliferative disorders [1,2]. The NF-κB is a transcription factor that functions as hetero- or homo-dimeric forms of Rel family proteins such as RelA (p65), RelB, cRel, p50 and p52 [3]. Under normal conditions, NF-κB is sequestered in the cytoplasm as an inactive complex, bound to inhibitory κB (IκB) proteins [4]. In response to lipopolysaccharide (LPS), an endotoxin recognized by toll-like receptor (TLR)-4/myeloid differentiation protein-2 complex on the immune cells, IκB kinase (IKK) is

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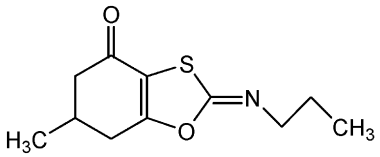

Fig. 1 – Chemical structure of iminobenzoxathiolone LYR-71.

activated and its Ser/Thr kinase activity phosphorylates the cytoplasmic IκB proteins [5,6]. The phosphorylated forms of IκBs are subjected to ubiquitination and then degraded in the proteasome [7]. Upon IκB degradation, NF-κB moves into the nucleus and then binds to the κB motif of inflammatory and immune genes, including interleukin (IL)-1β and other cytokines, for transcriptional activation [8,9].

IKK complex consists of IKKα, IKKβ and NEMO/IKKγ subunits. IKKα and IKKβ possess catalytic activity of Ser/Thr kinase, and NEMO/IKKγ functions as a regulatory subunit of the complex. Genetic study of IKK subunit-knockout mice has revealed that most of the inflammatory stimuli, including LPS, require the IKKβ subunit for NF-κB activation [10]. Dominant-negative IKKβ, but not dominant-negative IKKα, blocks NF-κB-regulated gene transcription in the inflammatory states [11]. However, IKKα has evidenced to play an important role in the embryonic developments [12].

Benzoxathiolone derivatives have been used in the treatment of acne and reported to have antipsoriatic and antibacterial properties [13,14]. However, the molecular mechanism of these pharmacological properties remains to be determined, even though NF-κB activation is closely linked to the inflammatory and immune diseases [1,3]. In this study, novel iminobenzoxathiolone derivative (LYR-71) of 6-methyl-2-propylimino-6,7-dihydro-5*H*-benzo[1,3]oxathiol-4-one (Fig. 1) has been demonstrated as an efficient inhibitor of IKKβ, preventing NF-κB activation in macrophages, and this mechanism of action contributed its down-regulatory effect on LPS-induced transcription of interleukin (IL)-1β or other cytokines.

2. Experimental procedures

2.1. Materials

Fetal bovine serum (FBS) and culture media were purchased from Invitrogen (Carlsbad, CA, USA). Antibodies against IKKβ or IκBα were obtained from Santa Cruz Biotech (Santa Cruz, CA, USA), and antibody against phosphor (p)-IκBα from Cell Signaling Tech (Danvers, MA). pNF-κB-secretory alkaline phosphatase (SEAP) construct was obtained from BD Biosciences Clontech (Palo Alto, CA, USA), expression vectors encoding IKKβ, NF-κB p65 or p50 from Dr. J.H. Lee (Kangwon National University, Chunchon, Korea), and pIL-1β-luciferase (Luc) construct from Dr. A. Aderem (Osaka University, Osaka, Japan). All other chemicals including LPS (*Escherichia coli* 055:B5) and zymosan (*Saccharomyces cerevisiae*) were otherwise purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Chemical preparation of LYR-71

Rhodium acetate (4.4 mg, 0.01 mmol) was added to a solution of 2-diazo-5-methyl-cyclohexane-1,3-dione (152 mg, 1.0 mmol) and propyl isothiocyanate (202 mg, 2.0 mmol) in fluorobenzene (2 ml), and stirred for 6 h at room temperature. After evaporating solvent in the reaction mixture under reduced pressure, a residue was subjected to a flash column chromatography on silica gel, eluting with *n*-hexane/ethyl acetate (2:1), to obtain LYR-71 (>97% purity, 113 mg). Spectral data of LYR-71: ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.08 (2H, t, J = 6.8 Hz), 2.82 (1H, dd, J = 13, 6.6 Hz), 2.61 (1H, d, J = 16.2 Hz), 2.44–2.37 (2H, m), 2.31–2.22 (1H, m), 1.73–1.66 (2H, m), 1.18 (3H, d, J = 5.9 Hz), 0.98 (3H, t, J = 7.3 Hz); IR 2961, 2932, 2875, 1684, 1624, 1457, 1364, 1329, 1223, 1097, 1077, 1019, 968, 952, 783 cm^{-1}.

2.3. Cell culture

RAW 264.7 or THP-1 cells are murine or human macrophages, respectively, and were purchased from American Type Culture Collection (Manassas, VA, USA). They were grown in Dulbecco's modified Eagle's media supplemented with 10% FBS, benzylpenicillin potassium (143 U/ml) and streptomycin sulfate (100 μg/ml) under 37 °C and 5% CO_2 atmosphere. RAW 264.7 cells harboring pNF-κB-SEAP-neomycin phosphotransferase (NPT) construct were obtained from Dr. Y.S. Kim (Seoul National University, Seoul, Korea), and cultured in the same conditions except supplement of geneticin (500 μg/ml) to the media.

2.4. Enzyme assay of IKKβ

A purified IKKβ (specific activity 20,000 U/mg protein) of human origin was obtained from Millipore Corp. (Billerica, MA, USA), in which one unit of the enzyme equals the incorporation of 1 pmol of phosphate into IκB substrate (100 μM) per min, at 30 °C with ATP (100 μM). Ser/Thr kinase activity of the IKKβ (usually 100–200 ng) was measured using a fluorescence resonance energy transfer (FRET)-based assay kit (Invitrogen, Carlsbad, CA, USA). In another, cell extracts (200 μg protein) of macrophages RAW 264.7 were incubated with anti-IKKβ antibody (2 μg) and protein A bead (GE Healthcare, Buckinghamshire, UK) at 4 °C overnight. Catalytic activity of the immunoprecipitated IKKβ was measured as described previously [15]. Briefly, enzyme sources were incubated with GST-IκBα (2 μg) as the substrate and [γ-^{32}P]ATP (5 μCi) in a reaction buffer (20 mM HEPES, pH 7.7, 2 mM $MgCl_2$, 50 mM ATP, 10 mM β-glycerophosphate, 10 mM NaF, 300 μg/ml Na_3VO_4, 2 μM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM dithiothreitol) at 30 °C for 1 h. The reaction mixtures were resolved on SDS-acrylamide gel by electrophoresis, and then subjected to autoradiography.

2.5. Western blot analysis

Macrophages RAW 264.7 were treated with LYR-71 for 2 h and stimulated with LPS (1 μg/ml) for 10 min (*p*-IκBα) or 30 min (total IκBα). Cell extracts were subjected to Western blot

analysis as described previously [16]. Briefly, equal amounts of protein were resolved on SDS-acrylamide gel by electrophoresis and transferred to PVDF membrane. Either 5% non-fat milk in phosphate-buffered saline with Tween 20 or 5% bovine serum albumin in Tris-buffered saline with Tween 20 was used as the blocking buffer. The blots were incubated at 4 °C overnight with primary antisera of anti-IκBα (1:1000) or anti-*p*-IκBα (1:300). The blots were then incubated at room temperature for 2 h with horseradish-labeled secondary antisera. Immune complexes on the blots were visualized by exposure to X-ray film after reacting with an enhanced chemiluminescence reagent (GE Healthcare, Buckinghamshire, UK).

2.6. SEAP assay

Macrophages RAW 264.7 harboring pNF-κB-SEAP-NPT reporter construct were treated with LYR-71 for 2 h and stimulated with LPS (1 μg/ml) for 16 h. Macrophages THP-1 were transiently transfected with pNF-κB-SEAP reporter construct (BD Biosciences Clontech, Palo Alto, CA, USA), using Lipofectamine (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendation. After allowing overnight to recover, the transfected cells were with LYR-71 for 2 h and stimulated with LPS (1 μg/ml) or water-insoluble zymosan (0.3 mg/ml) for 16 h. SEAP activity was measured as described previously [17]. Briefly, aliquots of the culture media were heated at 65 °C for 10 min, and then reacted with 4-methylumbelliferyl phosphate (500 μM) in the dark at room temperature for 1 h. SEAP activity was measured as relative fluorescence units (RFU) with emission at 449 nm and excitation at 360 nm. In another, RAW 264.7 cells harboring pNF-κB-SEAP-NPT reporter construct were transiently transfected with pSV-β-galactosidase control vector, in combination with expression vectors encoding IKKβ, NF-κB p65 or p50, using the Lipofectamine. After allowing overnight to recover, the transfected cells were treated with LYR-71 for 16 h, and then subjected to the SEAP assay.

2.7. Enzyme-linked immunosorbent assay (ELISA)

Macrophages RAW 264.7 were treated with LYR-71 for 2 h and stimulated with LPS (1 μg/ml) for 24 h. Amounts of IL-1β or TNF-α in the culture media were measured, using appropriate ELISA kits (R&D Systems, Minneapolis, MN, USA).

2.8. Reverse transcription-polymerase chain reaction (RT-PCR)

Macrophages RAW 264.7 were treated with LYR-71 for 2 h and stimulated with LPS (1 μg/ml) for 6 h. Total RNA of the cells was subjected to semi-quantitative RT-PCR, using an RNA PCR kit (Bioneer Co., Daejeon, Korea). Briefly, total RNA was reversely transcribed at 42 °C for 1 h, and then subjected to 25–30 cycles of PCR consisting of 30-s denaturation at 94 °C, 30-s annealing at 50–60 °C and 90-s extension at 72 °C. Oligonucleotides used for amplification and size of the PCR products are described in our previous work [18]. RT-PCR products were finally resolved on agarose gel by electrophoresis and stained with ethidium bromide.

2.9. Luciferase assay

Macrophages RAW 264.7 were transiently transfected with pIL-1β-Luc reporter construct and pSV-β-galactosidase control vector, using Lipofectamine according to the manufacturer's recommendation. After allowing overnight to recover, the transfected cells were treated with LYR-71 for 2 h and stimulated with LPS (1 μg/ml) for 16 h. Cell extracts were subjected to luciferase and β-galactosidase assays using appropriate kits (Promega, Madison, WI, USA). In another, the cells were transiently transfected with pIL-1β-Luc reporter construct and pSV-β-galactosidase control vector, in combination with expression vector encoding IKKβ, using the Lipofectamine. After allowing overnight to recover, the transfected cells were treated with LYR-71 for 16 h, and then subjected to the luciferase assay.

2.10. Analysis of cell viability

Macrophages RAW 264.7 were treated with various concentrations of LYR-71 for 24 h, exposed to a water-soluble WST-1 of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-terazolium (Dojindo Laboratories, Kumamoto, Japan) for 3 h, and then measured absorbance values at 450 nm.

2.11. Statistical analysis

Data are expressed as means ± S.E.M., and were analyzed by ANOVA followed by the Dunnet's test. Different values of $P < 0.01$ were considered significant.

3. Results

3.1. Novel iminobenzoxathiolone LYR-71 was an efficient inhibitor of IKKβ

IKKβ-mediated phosphorylation of cytoplasmic IκBs is a key event required for NF-κB activation in macrophages stimulated with inflammatory stimuli, including LPS [19]. First, we investigated whether LYR-71 (Fig. 1) could affect Ser/Thr kinase activity of a purified IKKβ of human origin, using a FRET-based assay. Enzyme IKKβ was incubated with LYR-71 for 20 min before the kinase reaction. LYR-71 inhibited Ser/Thr kinase activity of IKKβ in a dose-dependent manner, corresponding to 28% inhibition at 3 μM, 66% at 10 μM and 85% at 30 μM with an IC_{50} value of 7 μM (Fig. 2A). However, LYR-71 could not show significant inhibitory effect on the Ser/Thr kinase activity of IKKβ, when the compound was treated to the enzyme in the presence of FRET-based substrate (Fig. 2A). Catalytic activity of human IKKβ was also determined by the phosphorylation of GST-IκBα as the substrate. LYR-71 inhibited the purified IKKβ-catalyzed GST-IκBα phosphorylation *in vitro*, only when the compound was pre-incubated with the enzyme for 20 min before the kinase reaction (Fig. 2B). To better understand the time it takes for LYR-71 to inactivate IKKβ, we carried out a time-course study. LYR-71 (30 μM) exhibited 85–90% inhibitions on the catalytic activity of human IKKβ at pre-incubation for 10–20 min with the enzyme, significantly low 54% inhibition at pre-incubation for 5 min,

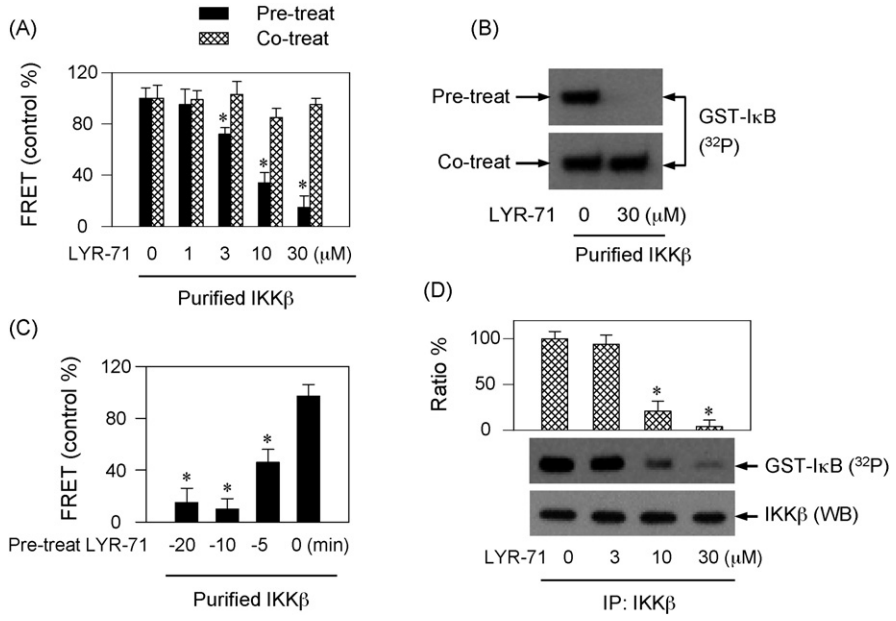

Fig. 2 – Catalytic activity of IKKβ. A purified IKKβ of human origin was pre-incubated with LYR-71 for 20 min before the kinase reaction (pre-treat) or reacted with LYR-71 in the presence of substrate (co-treat). (A) Ser/Thr kinase activity of the purified IKKβ was measured by a FRET-based assay and is represented as control percentage, compared with enzyme alone-treated group. (B) Catalytic activity of the purified IKKβ was also measured by phosphorylation (^{32}P) of GST-IκB as the substrate. (C) A purified human IKKβ was pre-incubated with LYR-71 for the indicated times, and its activity was then measured by FRET-based assay. Effect of LYR-71 on the IKKβ activity is represented as control percentage, compared with enzyme alone-treated group. (D) Cell extracts of macrophages RAW 264.7 were immunoprecipitated with anti-IKKβ antibody. The immunoprecipitated IKKβ (IP: IKKβ) was pre-incubated with LYR-71 for 20 min, and then subjected to the kinase assay of GST-IκB phosphorylation (^{32}P), and to Western blot analysis (WB). One of similar results is represented and relative ratio % is also indicated, in which GST-IκB signal was normalized to IKKβ signal. Data are means ± S.E.M. from 3 to 5 separate experiments. (*) $P < 0.01$ vs. enzyme alone-treated group.

and no inhibition at co-treatment in the presence of substrate (Fig. 2C).

We next determined whether LYR-71 could affect catalytic activity of IKKβ of murine origin. Macrophages RAW 264.7 were stimulated with LPS alone for 10 min, and then subjected to immunoprecipitation with anti-IKKβ antibody. The immunoprecipitated IKKβ still exhibited catalytic activity of GST-IκBα phosphorylation (Fig. 2D). Upon pre-incubation with the enzyme source for 20 min before kinase reaction, LYR-71 inhibited the *in vitro* GST-IκBα phosphorylation as a catalytic activity of murine IKKβ in a dose-dependent manner, corresponding to 6% inhibition at 3 μM, 79% at 10 μM and 96% at 30 μM (Fig. 2D).

3.2. *LYR-71 inhibited NF-κB activation in LPS-stimulated macrophages*

To understand whether LYR-71 could be also capable of inhibiting IKKβ activity in intact cells, we next performed Western blot analysis with anti-p-IκBα antibody, using macrophages RAW 264.7 stimulated with LPS in the absence and presence of LYR-71. As shown in Fig. 3A, phosphorylated forms of IκBα were hardly detectable in the normal cells. However, upon exposure to LPS alone for 10 min, cellular IκBα was markedly phosphorylated but its degradation had not started yet. LYR-71 dose-dependently inhibited LPS-induced IκBα phosphorylation in the RAW 264.7 cells (Fig. 3A).

The phosphorylated forms of cellular IκBs are subjected to ubiquitination followed by proteasome-mediated degradation [7,20]. Upon exposure to LPS alone, cellular IκBα was almost completely degraded within 30 min (Fig. 3B). LYR-71 dose-dependently prevented LPS-induced IκBα degradation in the RAW 264.7 cells (Fig. 3B).

The degradation of cellular IκBs is prerequisite to the nuclear translocation and transcriptional activation of NF-κB [8]. We examined further whether LYR-71 could inhibit NF-κB activation, using RAW 264.7 cells harboring pNF-κB-SEAP-NPT construct, containing four copies of the κB sequence fused to SEAP gene as a reporter [17]. Upon exposure to LPS alone, the cells increased SEAP expression up to 5- to 6-fold over the basal levels (Fig. 3C), indicating that cellular NF-κB is transcriptionally functional. LYR-71 inhibited LPS-induced SEAP expression in a dose-dependent manner, corresponding to 40% inhibition at 3 μM, 71% at 10 μM and 99% at 30 μM (Fig. 3C). To understand whether LYR-71 could affect NF-κB activation in human cells, macrophages THP-1 were transiently transfected with pNF-κB-SEAP construct. Consistently, LYR-71 inhibited LPS or water-insoluble zymosan-induced SEAP expression with IC_{50} values of 6-8 μM (Fig. 3D), in which water-insoluble zymosan substances are recognized by TLR-2

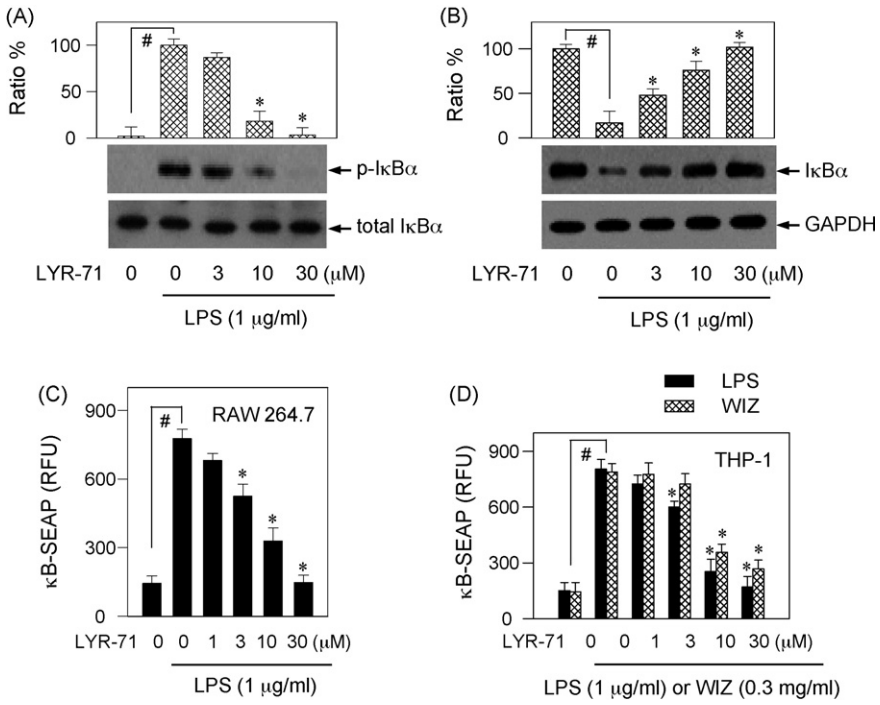

Fig. 3 – LPS-induced NF-κB activation. Macrophages RAW 264.7 were pretreated with LYR-71 for 2 h and stimulated with LPS for 10 min (A) or 30 min (B) in the presence of LYR-71. (A) Cell extracts were subjected to Western blot analysis with anti-p-IκBα antibody or anti-IκBα antibody. One of similar results is represented and relative ratio % is also indicated, in which p-IκBα signal was normalized to total IκBα signal. (B) Cell extracts were subjected to Western blot analysis with anti-IκBα antibody or anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody. One of similar results is represented and relative ratio % is also indicated, in which IκBα signal was normalized to GAPDH signal. (C) RAW 264.7 cells harboring pNF-κB-SEAP-NPT construct were pretreated with LYR-71 for 2 h and stimulated with LPS for 16 h, in the presence of LYR-71. As the reporter of NF-κB transcriptional activity, SEAP expression was measured as relative fluorescence units (RFU). (D) THP-1 cells were transiently transfected with pNF-κB-SEAP construct (1 μg/ml) and pSV-β-galactosidase control vector (1 μg/ml), using Lipofectamine according to the manufacturer's recommendation. After allowing overnight to recover, the transfected cells were pretreated with LYR-71 for 2 h and stimulated with LPS or water-insoluble zymosan (WIZ) for 16 h, in the presence of LYR-71. SEAP expression was measured as relative fluorescence units (RFU) and then normalized to β-galactosidase activity. Data are means ± S.E.M. from three separate experiments. (#) $P < 0.01$ vs. media alone-treated group. (*) $P < 0.01$ vs. LPS alone- or WIZ alone-treated group.

and Dectin-1 receptors on macrophages for NF-κB activation [21].

3.3. *LYR-71 inhibited cellular NF-κB activation elicited by expression vector IKKβ but not by expression vector NF-κB p65 or p50*

As described above, LYR-71 was an efficient inhibitor of IKKβ activity *in vitro* (Fig. 2), and inhibited the phosphorylation and degradation of cellular IκBα as well as the NF-κB transcriptional activity in macrophages (Fig. 3). To confirm the cellular target of LYR-71, we transfected RAW 264.7 cells harboring pNF-κB-SEAP-NPT reporter construct with expression vectors encoding IKKβ, NF-κB p65 or p50, and then examined the effect of LYR-71 directly on the resultant NF-κB activation. Transfection with IKKβ vector resulted in a significantly increased expression of SEAP reporter in the cells, which was inhibited by treatment of LYR-71 in a dose-dependent manner (Fig. 4A). In contrast, LYR-71 could not significantly affect NF-κB p65 or p50 vector-elicited SEAP expression in the cells (Fig. 4B and C). These results indicate that a target event of LYR-71 could be upstream IκB degradation in the NF-κB activating pathway, specifically IKKβ-catalyzed IκBα phosphorylation in macrophages.

3.4. *LYR-71 down-regulated NF-κB-dependent expression of IL-1β or other cytokines*

NF-κB is a transcription factor that regulates the expression of diverse inflammatory genes, and has been evidenced to play a major mechanism in the transcriptional activation of LPS-inducible IL-1β gene in macrophages [9,22–24]. Macrophages RAW 264.7, in the normal state, released 100–184 pg/ml of IL-1β during incubation for 24 h, and markedly increased IL-1β levels to 1925–2083 pg/ml, upon exposure to LPS alone (Table 1). LYR-71 inhibited LPS-induced IL-1β production in a dose-dependent manner, corresponding to 6% inhibition at 3 μM, 51% at 10 μM and 93% at 30 μM (Table 1). In a parallel experiment, TNF-α levels were increased to 72–83 ng/ml from the basal levels of 6–10 ng/ml, upon exposure to LPS alone,

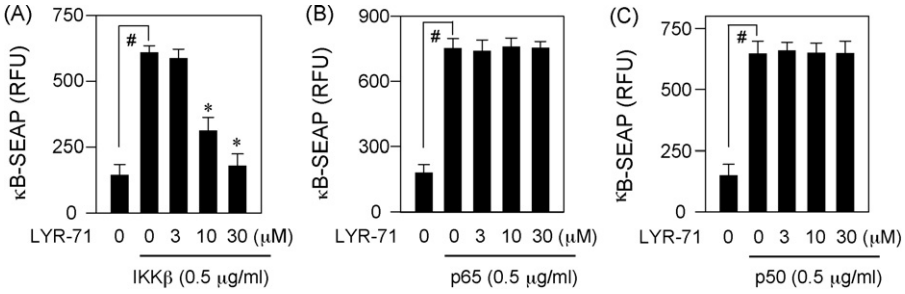

Fig. 4 – Cellular NF-κB activation elicited by expression vectors encoding IKKβ, NF-κB p65 or p50. Macrophages RAW 264.7 harboring pNF-κB-SEAP-NPT construct were transiently transfected with pSV-β-galactosidase control vector (1 μg/ml), in combination with each expression vector (0.5 μg/ml) encoding IKKβ (A), NF-κB p65 (B) or p50 (C), using Lipofectamine according to the manufacturer's recommendation. After allowing overnight to recover, the transfected cells were treated with LYR-71 for 16 h. SEAP activity in the culture media was measured as relative fluorescence units (RFU) and then normalized to β-galactosidase activity. Data are means ± S.E.M. from three separate experiments. (#) $P < 0.01$ vs. pSV-β-galactosidase control vector alone-transfected group. (*) $P < 0.01$ vs. each expression vector alone-transfected group.

which was also inhibited by treatment of LYR-71 (Table 1). However, LYR-71 at the effective concentrations did not affect the proliferation of RAW 264.7 cells (Fig. 5), indicating that its suppressive effects on cytokine production could not be attributable to non-specific cytotoxicity.

To understand whether inhibitory action of LYR-71 on LPS-induced expression of inflammatory cytokines had taken place at the transcription level, semi-quantitative RT-PCR was carried out. IL-1β transcript was hardly detectable in the normal cells, and markedly increased, upon exposure to LPS alone (Fig. 6A). LYR-71 dose-dependently attenuated LPS-induced synthesis of IL-1β transcript in the cells (Fig. 6A). In a parallel experiment, LPS-induced synthesis of TNF-α or IL-6 transcript was also inhibited by treatment of LYR-71 (Fig. 7).

Transcriptional control of IL-1β expression by LYR-71 was further demonstrated by a promoter activity assay, using RAW 264.7 cells transfected with pIL-1β-Luc construct encoding IL-1β promoter (−1856/+1) fused to luciferase gene as a reporter [24]. Upon exposure to LPS alone, the transfected cells increased luciferase expression up to about 40-fold over the basal levels (Fig. 6B). LYR-71 inhibited LPS-induced luciferase expression in a dose-dependent manner (Fig. 6B). These results indicate that LYR-71 could down-regulate LPS-induced IL-1β expression at the transcription level.

Since LYR-71 inhibited LPS-induced NF-κB activation (Fig. 3) and also down-regulated LPS-induced IL-1β expression at the transcription level (Fig. 6A and B), we decided to demonstrate whether LYR-71 could directly affect NF-κB-dependent expression of IL-1β. Macrophages RAW 264.7 were transfected with pIL-1β-Luc reporter construct, in combination with expression vector encoding IKKβ. Luciferase expression as a reporter of IL-1β promoter activity was efficiently increased, upon transfecting the cells with expression vector IKKβ (Fig. 6C). LYR-71 inhibited IKKβ vector-elicited luciferase expression in a dose-dependent manner (Fig. 6C).

Table 1 – Effect of LYR-71 on LPS-induced production of IL-1β or TNF-α

Treatment	IL-1β (pg/ml)	TNF-α (ng/ml)
Media alone	142 ± 42	8 ± 2
LPS alone	2004 ± 79[a]	77 ± 5[a]
LPS + LYR-71 (1 μM)	1932 ± 125	75 ± 6
LPS + LYR-71 (3 μM)	1902 ± 151	70 ± 8
LPS + LYR-71 (10 μM)	1027 ± 41[b]	56 ± 5[b]
LPS + LYR-71 (30 μM)	236 ± 17[b]	20 ± 3[b]

Macrophages RAW 264.7 were pretreated with LYR-71 for 2 h and stimulated with LPS (1 μg/ml) for 24 h, in the presence of LYR-71. Amounts of the cytokines were measured in the culture media by ELISA. Data are means ± S.E.M. from three separate experiments.
[a] $P < 0.01$ vs. media alone-treated group.
[b] $P < 0.01$ vs. LPS alone-treated group.

4. Discussion

Molecular basis of multiple pharmacological properties assigned to benzoxathiolone derivatives has not been defined, even though NF-κB activation is closely linked to the

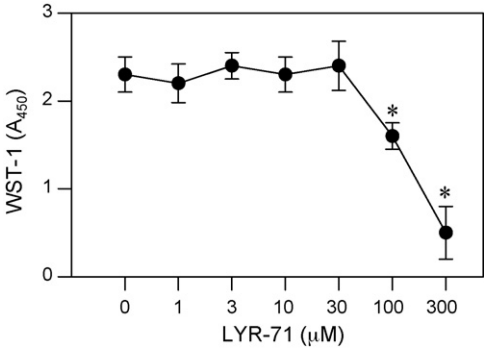

Fig. 5 – Proliferation of RAW 264.7 cells. The cells were incubated with various concentrations (1–300 μM) of LYR-71 for 24 h. They were treated with WST-1 solution for 3 h, and then measured absorbance values at 450 nm (A_{450}). Data are means ± S.E.M. from three separate experiments. (*) $P < 0.01$ vs. media alone-treated group.

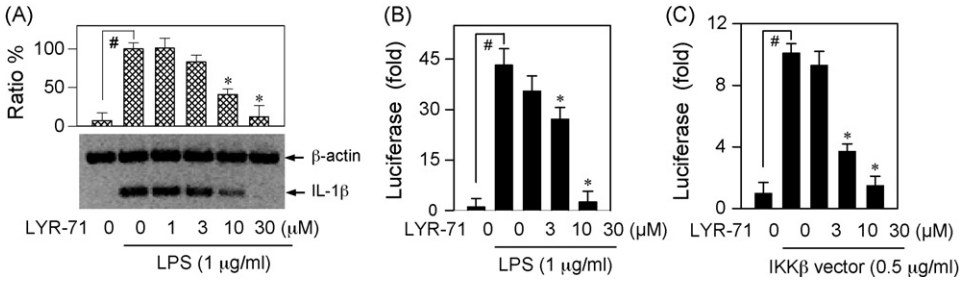

Fig. 6 – LPS or IKKβ vector-induced IL-1β expression. (A) Macrophages RAW 264.7 were pretreated with LYR-71 for 2 h and stimulated with LPS for 6 h, in the presence of LYR-71. Total RNA of the cells was subjected to semi-quantitative RT-PCR. One of similar results is represented and relative ratio % is also indicated, in which IL-1β signal was normalized to β-actin signal. (B) RAW 264.7 cells were transiently transfected with pIL-1β-Luc reporter construct (1 μg/ml) and pSV-β-galactosidase control vector (1 μg/ml), using Lipofectamine according to the manufacturer's recommendation. After allowing overnight to recover, the transfected cells were pretreated with LYR-71 for 2 h and stimulated with LPS for 16 h, in the presence of LYR-71. Luciferase expression, a reporter of IL-1β promoter activity, was measured with cell extracts and is represented as relative fold, in which luciferase activity was normalized to β-galactosidase activity. (C) RAW 264.7 cells were transiently transfected with pIL-1β-Luc reporter construct (1 μg/ml) and pSV-β-galactosidase control vector (1 μg/ml), in combination with expression vector encoding IKKβ (0.5 μg/ml), using Lipofectamine according to the manufacturer's recommendation. After allowing overnight to recover, the transfected cells were treated with LYR-71 for 16 h. Luciferase expression was measured with cell extracts and is represented as relative fold, in which luciferase activity was normalized to β-galactosidase activity. Data are means ± S.E.M. from three separate experiments. (#) $P < 0.01$ vs. media alone-treated group (A and B) or pIL-1β-Luc reporter construct alone-transfected group (C). (*) $P < 0.01$ vs. LPS alone-treated group (A and B) or pIL-1β-Luc reporter construct plus IKKβ vector alone-transfected group (C).

inflammatory and immune disorders. A goal of this study is to elucidate a target molecule of LYR-71 (Fig. 1) on NF-κB activation, and to demonstrate its effect on LPS-induced expression of inflammatory cytokines in macrophages.

In the present study, LYR-71 was discovered as an efficient inhibitor of IKKβ, a key enzyme in the NF-κB activating pathway (Fig. 2). LYR-71 inhibited catalytic activity of human or murine IKKβ, only when the compound was pre-incubated with enzyme sources before the kinase reaction (Fig. 2), suggesting that LYR-71 would be slowly reacting with IKKβ to inactivate the enzyme activity. Consistently, LYR-71 inhibited LPS-induced IκBα phosphorylation in RAW 264.7 cells (Fig. 3A), and sequentially preventing IκBα degradation (Fig. 3B) as well as NF-κB transcriptional activity (Fig. 3C). Moreover, LYR-71 inhibited NF-κB activation in human cell line, macrophages THP-1 stimulated with LPS or water-insoluble zymosan (Fig. 3D).

To elucidate a target event of LYR-71, we transfected RAW 264.7 cells harboring pNF-κB-SEAP-NPT reporter construct with each of expression vectors encoding IKKβ, NF-κB p65 or p50. LYR-71 inhibited cellular NF-κB activation elicited by IKKβ vector, but not that resultant from transfection of other vectors encoding NF-κB p65 or p50 (Fig. 4). Therefore, LYR-71 prevented LPS-induced NF-κB activation in macrophages targeting upstream IκBα degradation, specifically IκBα phosphorylation through its inhibitory mechanism on catalytic activity of IKKβ.

Recently, 6,6,-dimethyl-2-(phenylimino)-6,7-dihydro-5*H*-benzo[1,3]oxathiol-4-one (BOT-64) has been reported to block NF-κB activation with an IC_{50} value of 1 μM by a Michael-type addition to the activation loop of IKKβ, resulting in inhibition of the enzyme activity [25]. A phenylimino group of BOT-64

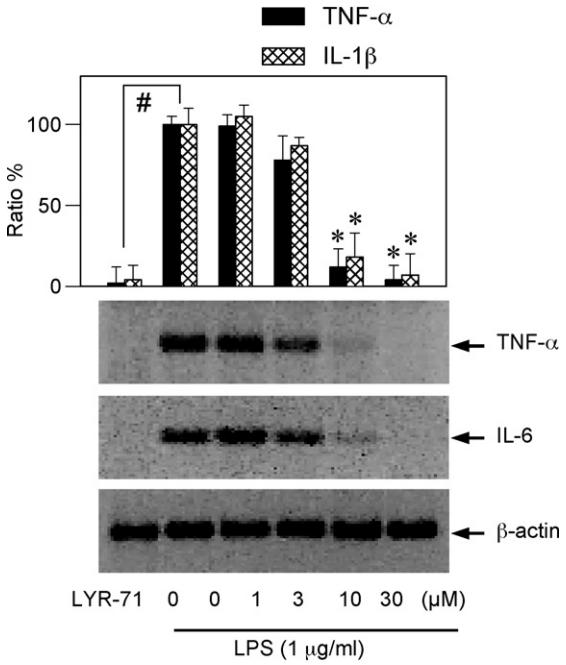

Fig. 7 – RT-PCR of TNF-α or IL-6 transcript. RAW 264.7 cells were pretreated with LYR-71 for 2 h and stimulated with LPS for 6 h, in the presence of LYR-71. Total RNA of the cells was subjected to semi-quantitative RT-PCR. One of similar results is represented and relative ratio % is also indicated, in which TNF-α or IL-6 signal was normalized to β-actin signal. Data are means ± S.E.M. from three separate experiments. (#) $P < 0.01$ vs. media alone-treated group. (*) $P < 0.01$ vs. LPS alone-treated group.

was modified with a propylimino group in the present LYR-71. We expected that this replacement could make the nitrogen on imino group to be more basic, coming to protonation at physiological condition, and also the β-carbon on α,β-unsaturated carbonyl structure to be more electron deficient, enhancing reactivity to the nucleophile residues of IKKβ. LYR-71 was more water soluble than BOT-64. However, this replacement unfortunately decreased the inhibitory potency on cellular NF-κB activation (Fig. 3). The structure-activity relationship of iminobenzoxathiolone compound will be delineated in a future study.

Since IKKβ-mediated phosphorylation of cytoplasmic IκBs represents a convergent point for most pathogenic stimuli leading to NF-κB activation, IKKβ is widely considered as a key target for the development of NF-κB inhibitor with pharmacological potential in inflammatory and immune disorders [1,19]. Some of non-steroidal anti-inflammatory drugs such as aspirin and sulindac have been reported to inhibit catalytic activity of IKKβ by a competitive mechanism on ATP binding to the enzyme [26,27]. Thiol-reacting drugs such as arsenic trioxide, at present under clinical trials for treatment of leukemia and solid tumors, and the gold compound auranofin alter cysteine-179 residue in the activation loop of IKKβ, resulting in inhibition of NF-κB activation [28,29]. Epoxyquinoid derivatives such as manumycin A and jesterone dimer are another class of IKKβ inhibitors that induce a covalent dimerization of IKKβ, preventing the association of NEMO/IKKγ with the IKKα and IKKβ [30,31]. Selective inhibitors of IKK complex, which target preferentially IKKβ over IKKα, have been recently developed by the pharmaceutical industry. Some of them, including β-carboline PS-1145 and imidazoquinoxaline BMS-345541, have undergone preclinical studies for the treatment of arthritic and proliferative disorders [32,33].

To investigate the influence of LYR-71 on NF-κB-regulated gene transcription, we demonstrated that LYR-71 could inhibit LPS-induced production of inflammatory cytokines in macrophages RAW 264.7 (Table 1). Furthermore, LYR-71 differentially attenuated LPS-induced synthesis of IL-1β, TNF-α or IL-6 transcript in the cells (Figs. 6A and 7), as well as inhibited LPS-induced IL-1β promoter activity (Fig. 6B). These results indicate that LYR-71 could down-regulate LPS-induced expression of IL-1β or other cytokines at the transcription level. A number of *cis*-acting regulatory elements have been reported in the IL-1β promoter, including two LPS-responsive κB sites at −2800/−2720 and −296/−286 [34]. Since NF-κB has been evidenced to play a pivotal role in the transcription of LPS-inducible IL-1β gene in macrophages, we further demonstrated that LYR-71 could inhibit IL-1β promoter activity elicited directly by transfecting expression vector IKKβ (Fig. 6C).

In conclusion, iminobenzoxathiolone LYR-71 is a small-molecule inhibitor of IKKβ activity, preventing NF-κB activation in macrophages. This mechanism of action could contribute to suppressive effect of LYR-71 on NF-κB-regulated transcription of inflammatory cytokines in the cells. Taken together, this study provides a molecular basis of pharmacological properties assigned to benzoxathiolone derivatives, and also anti-inflammatory potential of LYR-71 in the NF-κB-activated inflammatory disorders.

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